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Structural and Functional Characterization of Plasma Fibronectin in Patients with Essential Mixed Cryoglobulinaemia

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Summary: Experimental studies suggest that plasma fibronectin may be involved in the cryoprecipitation of cryoglobulins in essential mixed cryoglobulinaemia; reduced plasma concentrations of the glycoprotein have been shown in the disease. The present work was undertaken in order to verify this latter finding and to detect a possible structural alteration of plasma fibronectin as result of enzymatic digestion of the molecule in vivo. This could, in turn, induce a decreased reactivity of the protein in immunometric assays and a reduced opsonic activity, which is normally due to the affinity of fibronectin to the C1q component of complement. Moreover, since a polymorphic variant of fibronectin has been described in plasma during experimental vascular injury and in patients with autoimmune vascular diseases, the aim of this study was also to verify the presence of a polymorphism of the glycoprotein in cryoglobulinaemic vasculitis. Twenty seven patients with essential mixed cryoglobulinaemia and 26 normal subjects were included in the study. Significantly reduced concentrations of plasma fibronectin, as assessed by ELISA, were found in patients when compared with controls (231.7 ± 15.3 vs 316.1 ± 16.6 mg/l, $P < 0.0002$). In contrast, when affinity-purified plasma fibronectin from 10 patients with essential mixed cryoglobulinaemia and 8 healthy subjects were analysed by western blotting, employing a panel of five monoclonal antibodies to different regions of the molecule, no differences were observed between patients and controls, suggesting integrity of the glycoprotein in the disease. Moreover, plasma fibronectin from cryoglobulinaemic patients and normal subjects bound to solid phase C1q in a dose-dependent manner with identical efficiency in the two groups, further suggesting that the molecule is functionally and structurally unaltered in the disease. The production of an abnormally glycosylated form of fibronectin in patients with essential mixed cryoglobulinaemia also seems to be excluded, as SDS-PAGE revealed no differences in electrophoretic mobility and apparent molecular weight between fibronectin from patients and controls. Taken together these data are consistent with the hypothesis that plasma fibronectin concentrations are actually reduced in essential mixed cryoglobulinaemia possibly by consumption during cryoprecipitate formation, and that a polymorphic form of the protein is not released into the circulation during cryoglobulinaemic vasculitis.

Introduction

Fibronectins are high-molecular-mass glycoproteins present in the soluble form in plasma and in insoluble form in the extracellular matrix and basement membranes (1). The fibronectin molecule is composed of multiple globular domains capable of distinct interactions with other biological structures including fibrin, heparin, plasma transglutaminase (factor XIIIa), *S. aureus*, collagen, DNA, cells and fibronectin itself; consequently it plays a central role in several biologic phenomena (2–4). Experimental studies have shown that fibronectin may be involved in some immune functions. It has been demonstrated that plasma fibronectin, mainly produced by hepatocytes (5), acts as a non-specific opsonin for particulate materials and microaggregates present in the circulation by

enhancing their clearance by the reticuloendothelial system (6–10). It has also been suggested that this glycoprotein may play a role in reticuloendothelial clearance of circulating immune-complexes primarily by binding, through its collagen binding domain, to the C1q component of complement (11–13).

Essential mixed cryoglobulinaemia is a disorder characterized by widespread vasculitis and multiple organ involvement mainly due to the deposition in small vessels and tissues of circulating cold-insoluble immune-complexes (cryoglobulins) composed of monoclonal IgM with rheumatoid-factor activity bound to polyclonal IgG (14–16). Experimental studies by us and others have shown that plasma fibronectin may be associated with cryoglobulins and that it may participate in cryoprecipitate formation (17–19). We also recently demonstrated that immunoenzymatically measured plasma fibronectin concentrations are reduced in patients with essential mixed cryoglobulinaemia suggesting that an impairment in plasma opsonic activity may be a feature of the disease and can therefore contribute to the abnormal persistence of cold-insoluble complexes in the circulation and to tissue damage (19, 20). However the possibility cannot be excluded that the reduced plasma fibronectin concentrations detected in cryoglobulinaemic patients are the result of an enzymatic cleavage of the molecule in vivo with loss of fragments essential for immunologic reactivity in the ELISA assays. This hypothesis seems to be supported by the demonstration by our group of increased concentrations of plasma tissue plasminogen activator and thrombin-antithrombin III complexes in these patients as a consequence of endothelial damage (20), suggesting that the fibronectin molecule is digested by plasmin produced by activation of the fibrinolytic pathway and/or by thrombin formed during the activation of the coagulation cascade (21–23).

Many reports on the molecular biology of fibronectin have demonstrated that, in different cell systems, a single fibronectin gene can give rise, by alternative splicing of the primary transcript, to several different mRNAs which in turn generate a family of different fibronectin molecules characterized by the differential expression of the number of homologous repeats within the functional domains (24–29). A fibronectin molecule containing an extra type III domain (ED1 + FN), absent in hepatocytes but present in endothelium and platelets, has been characterized (29–32), and has been detected in plasma during experimental blood vessel injury (30, 33) and in patients with active collagen vascular disorders (34). A polymorphism of plasma fibronectin associated to essential mixed cryoglobulinaemia has not so far been described. In the present study we measured plasma fibronectin concentrations in a larger series of patients with essential mixed cryoglobulinaemia and we used an analytical approach which employed a panel of five monoclonal antibodies to different functional domains of fibronectin molecule and the western blot technique to detect possible structural abnormalities of the glycoprotein in this condition. Plasma fibronectin binding to C1q in cryoglobulinaemic patients was also investigated.

Patients and Methods

Patients

Twenty seven patients (13 males and 14 females), aged 30 to 72 years (mean 55.2) and 26 normal subjects of comparable age (35–67 years, mean 53) and sex distribution (12 males and 14 females) were included in the study. The diagnosis of essential mixed cryoglobulinaemia was made after exclusion of underlying infectious, neoplastic, and systemic disorders. Quantitation and characterization of the cryoprecipitate were carried out as previously described (35). Mean cryocrit value was 15.2 ± 4.3% and the cryoglobulin composition was IgG-IgMk (monoclonal rheumatoid factor) in all cases. According to clinical chemical analyses, none of the patients showed any indications of hepatic failure. Nine patients were undergoing low-dose steroid therapy (prednisone 10–20 mg/day) at the time of the study. Some clinical and laboratory features of patients studied are shown in table 1.

Plasma samples

Fasting blood samples were drawn from patients and controls, after obtaining informed consent, into prewarmed plastic tubes containing 38 g/l sodium citrate; samples were immediately centrifuged at 2400 *g* for 15 min at 37 °C, and the plasma was collected and immediately deep frozen until assayed, or purified by chromatography. Before use samples were thawed at 37 °C to avoid fibrin formation.

Plasma fibronectin determination

This was performed with an enzyme-linked immunosorbent assay (ELISA) as previously described (19) which employed a goat IgG to human fibronectin (Jackson Laboratories) as first antibody (coating antibody) and a peroxidase-conjugated goat IgG to human fibronectin as second antibody. The standard curve was prepared using both commercially available purified fibronectin (Behring) and affinity-purified fibronectin prepared in our laboratory from normal human plasma (see below). Each immunoassay included patients and controls in each run. The plates were read in a multichannel photometer (Titertek Multiscan MC, Flow Laboratories) interfaced to an Apple IIe microcomputer and the results expressed in milligrams per litre. Between-day coefficient of variation was below 5% in both patients with essential mixed cryoglobulinaemia and controls.

Plasma fibronectin purification

Plasma fibronectin from 10 patients (5 men and 5 women) and 8 normal subjects (5 men and 3 women) was purified using gelatin affinity chromatography according to the method described by *Engvall & Rouslahti* (36). To prepare the gelatin affinity columns, gelatin (type I from porcine skin, Sigma) was coupled to CNBr-activated Sepharose 4B (Pharmacia) according to the procedure provided by the manufacturer. Citrated

Tab. 1. Some routine clinical and laboratory findings of patients with essential mixed cryoglobulinaemia. Complement levels were measured by radial immunodiffusion and values are expressed as % of reference normal plasma. Rheumatoid factor (RF) was assessed by ELISA and values are expressed as mg/l. Cryocrit is expressed as %. Reference values of our laboratory are 65–140% for C3, 43–156% for C4, 64–120% for C1q and below 8 mg/l for rheumatoid factor.

Patient	Age	Sex	Cryocrit	Characterization	C3	C4	C1q	RF
1	71	♂	6.3	IgG-IgMk	68	5	50	568
2	61	♀	4.6	IgG-IgMk	60	5	34	20
3	50	♀	8.8	IgG-IgMk	84	10	50	176
4	50	♂	6.5	IgG-IgMk	89	30	100	–
5	46	♂	15.2	IgG-IgMk	54	5	5	276
6	72	♂	9.3	IgG-IgMk	32	5	0	–
7	61	♂	23.0	IgG-IgMk	65	5	15	285
8	49	♀	11.6	IgG-IgMk	78	5	46	–
9	59	♀	60.0	IgG-IgMk	80	5	50	336
10	55	♀	24.4	IgG-IgMk	58	5	24	768

plasma (25 ml) was applied to the column and fibronectin was then eluted with 8 mol/l urea in 50 mmol/l Tris-buffer, pH 7.5. The entire procedure was performed at 37 °C. Protein was determined by absorbance at 280 nm, using as reference a standard curve prepared with commercially available purified fibronectin (Behring) at various dilutions. Plasma fibronectin recovery averaged 1.2 and 1.8 mg of protein in patients and controls respectively. Purity of plasma fibronectin preparations was assessed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (see results).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE of purified preparations was carried out under reducing conditions using 5% acrylamide slab gels (37). The samples were reduced by incubation with 20 g/l 2-mercapto-ethanol at 56 °C for 1 hour. Preliminary experiments were carried out by running purified plasma fibronectin preparations with reduced standard plasma fibronectin (Behring) (M_r 220 000), myosin (M_r 200 000), β-galactosidase (M_r 116 250), phosphorylase a (M_r 93 000), human serum albumin (M_r 68 000) and ovalbumin (M_r 45 000) as molecular mass markers (Biorad); gel slabs were stained with Coomassie blue.

Western blotting

Proteins were transferred electrophoretically from polyacrylamide gel onto nitrocellulose paper as described by *Towbin* et al. (38). The blots were soaked in Tris-NaCl pH 7.5 + 50 g/l bovine serum albumin overnight at room temperature, rinsed with phosphate buffered saline, containing 10 g/l bovine serum albumin and incubated with a 1 : 1000 dilution of the appropriate monoclonal antibody for 2 hours at 37 °C. After three extensive washes in phosphate buffered saline containing 10 g/l bovine serum albumin the blots were incubated with a 1 : 400 dilution of peroxidase-conjugated anti-mouse Ig antibody (Dako) for 1 hour at 37 °C and after three more washes the substrate (500 g/l 4-chloro-1-naphthol in H_2O_2) was added and sufficient time allowed for the reaction to occur.

Monoclonal antibodies

Five commercially available monoclonal antibodies (Mallinckrodt), each recognizing different human fibronectin epitopes, were used (39–43). Binding specificities and clone of origin of the antibodies employed are shown in table 2.

Plasma fibronectin binding to C1q

The binding of purified fibronectin from patients and controls to C1q was assessed by ELISA using a modification of the method previously described by *Baatrup & Svehag* (13). Briefly, the wells of microtitre plates (Dynatech) were coated overnight with a 4 mg/l solution of C1q (Sigma) in phosphate buffered saline. The plates were then washed three times with phosphate buffered saline Tween and blocked with phosphate buffered saline containing 1 g/l Tween and 0.5 g/l human serum albumin for 2 hours at room temperature. Purified plasma fibronectin samples were serially diluted in phosphate buffered saline, and 100 µl of the diluted samples and standards were added to each C1q-coated well in duplicate. The plates were then incubated for 2 hours at 37 °C. The standard fibronectin curve was obtained by using purified fibronectin from a commercial source

Tab. 2. Monoclonal antibodies employed in plasma fibronectin molecule mapping by western blotting. The specificity of the antibodies, i. e. the region of fibronectin recognized, and the clone from which the different monoclonal antibodies were isolated are reported accordingly with information specified in the data sheet provided by the manufacturer. For references see 39–43.

	Monoclonal antibody	Clone Immunoglobulin class	Specificity
CT	B68A11	IgG1	C-terminal domain
ECS	910D18	IgG1	elastase cleavage site
NT	1032B53	IgM	N-terminal domain
MM	875A51	IgG1	central portion of fibronectin monomer
CBP	784A2A6	IgG1	M_r 11 000 cell adhesive fragment
CBS	790D24	IgG1	pepsin cleavage site at N-terminal of the M_r 11 000 cell adhesive fragment

(Behring). After three more washes 100 µl of a peroxidase-conjugated goat anti-human fibronectin IgG (Jackson Laboratories) diluted 1 : 400 in phosphate buffered saline were added to each well. The plates were then incubated at 37 °C for 1 hour and washed. Finally the substrate (500 g/l ABTS in H_2O_2, KPL Laboratories) was added and sufficient time allowed for the reaction to occur. The plates were then read in a Titertek Multiscan MC photometer (Flow Laboratories) interfaced to an Apple IIe microcomputer.

Statistical analysis

Wilcoxon's rank sum test was applied when appropriate, using an IBM personal computer and a suitable statistical package (Epistat).

Results

The results of plasma fibronectin assay in patients with essential mixed cryoglobulinaemia and in controls are shown in figure 1. It can be seen that plasma fibronectin concentrations were significantly lower in the patients than in normal subjects (231.7 ± 15.3 vs 316.1 ± 16.6 mg/l, $P < 0.0002$), confirming our previous data obtained in a smaller group (20).

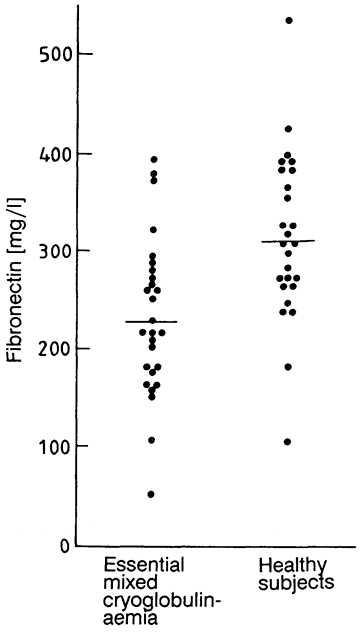

Fig. 1. Plasma fibronectin concentrations in patients with essential mixed cryoglobulinaemia (left) and in healthy subjects (right). p = 0.0002

In agreement with previously reported observations (36, 44), SDS-PAGE of reduced plasma fibronection from both patients and controls showed a single polypeptide band with a relative molecular mass of about M_r 250 000, indicating size homogeneity of the purified protein. No protein other than fibronectin was visualized by Coomassie blue staining in the purified preparations. Electrophoretic mobility of plasma fibronectin bands was identical in the two groups of subjects.

All purified plasma fibronectin samples from cryoglobulinaemic patients and control subjects stained with CBP, CBX, MM and CT monoclonal antibodies by western blot analysis (fig. 2). As can be seen, staining intensity and shape of the bands were comparable in the two groups, and no difference of reactivity was observed when commercially available plasma fibronectin (Behring) was tested. None of the plasma fibronectin preparations could be stained with the monoclonal antibody against N-terminal region of the molecule (NT), suggesting loss of the N-terminal peptide fragment during the chromatographic procedure.

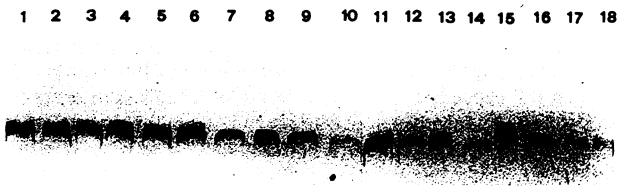

Fig. 2. Western blotting analysis of purified plasma fibronectin from normal subjects (lanes 1–4 and 15–18) and from patients with essential mixed cryoglobulinaemia (lanes 5–14) using 784A2A6 monoclonal antibody to the cell binding peptide of the fibronectin molecule.

The binding of plasma fibronectin from patients with essential mixed cryoglobulinaemia and controls to solid phase C1q is shown in figure 3. Purified fibronectin, tested at progressively halved dilutions in phosphate buffered saline at concentrations ranging from 4 to 500 mg/l, was found to bind in C1q coated wells in a dose-dependent fashion, thus confirming previously reported observations (11, 13). As can be observed, no significant difference in binding efficiency to C1q was observed between plasma fibronectin from cryoglobulinaemic patients and that from the control group.

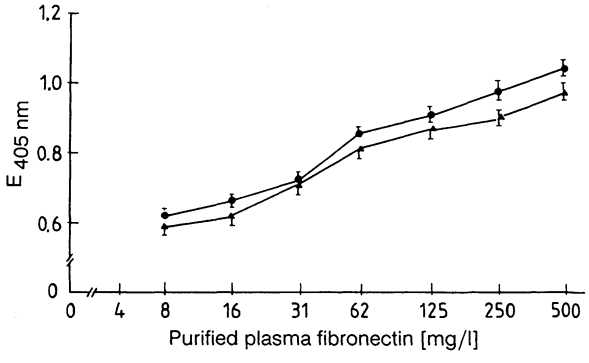

Fig. 3. Binding of purified plasma fibronectin from patients with essential mixed cryoglobulinaemia (▲) and from healthy subjects (●) to solid phase C1q. Data are expressed as mean ± SEM.

Discussion

In the present work we confirm that plasma fibronectin is significantly decreased in patients with essential mixed cryoglobulinaemia when compared with normal controls. In contrast, when western blotting was used to map the purified molecule obtained from both groups by a panel of five monoclonal antibodies to different fibronectin epitopes, no differences were revealed between fibronectin from patients and normal subjects, suggesting that the glycoprotein structure is unaltered in the disease. We previously showed that fibronectin is constantly present in cryoprecipitate from cryoglobulinaemic patients (19), suggesting that the molecule could be actively involved in the in vivo formation of the cryoprecipitate. This, in turn, could result in the consumption of the glycoprotein, leading to a reduction of its plasma concentration. The alternative hypothesis of an enzymatic cleavage of fibronectin by plasmin and/or thrombin produced in vivo in patients with essential mixed cryoglobulinaemia with loss of immunoreactive fragments, as suggested by recent data from our group (20), seems to be excluded on the basis of the western blotting results of the present study which demonstrate that plasma fibronectin concentrations are actually reduced in the disease.

It has been shown experimentally that in different cell systems different fibronectin mRNAs originate by alternative splicing of a common precursor RNA. These, in turn, give rise to a family of fibronectin polymorphic variants differing in their primary sequences (24–28). One of these polymorphic molecules containing an extra type III domain, ED1+FN, was detected in endothelium and platelets (29–32) and was shown to be released into the circulation during experimental vascular injury (30, 33) and to be increased in the plasma of patients with vasculitis associated with connective tissue disorders (34). In the same patients, an increased plasma concentration of total fibronectin was also reported. These data seem to contradict those found in the present work, since our experimental approach demonstrated no polymorphism of the glycoprotein in association with cryoglobulinaemic vasculitis, and significantly reduced rather than increased concentrations of total fibronectin were demonstrated in our patients. On the other hand, an impairment of the hepatic biosynthesis of plasma fibronectin, which can be advocated as a possible cause of the reduced plasma concentrations of the glycoprotein in essential mixed cryoglobulinaemia, may be ruled out by the normality of the liver function results constantly observed in our series.

We also demonstrated that plasma fibronectin binds to solid phase C1q in a dose-dependent manner, thus confirming previously reported observations (11, 13). Recently it was also clearly shown that the glycoprotein has a strong affinity for C1q bound to fluid phase immune-complexes (13, 45). This interaction, mediated by the M_r 50000 gelatin-binding domain of the molecule (46), may play a key role in immune-complex clearance by the reticuloendothelial system (12), which also critically depends on the interaction of the cell-binding domain of fibronectin with macrophages (42). On the other hand, fibronectin affinity to immune complex-associated C1q also seems to be important in mediating cryoprecipitation of cryoglobulins (11). Our data demonstrate that both domains are present in fibronectin purified from patients with essential mixed cryoglobulinaemia, suggesting that enzymatic cleavage of the molecule has not occurred at these sites and that opsonic function is retained by the glycoprotein in the disease. The demonstration that plasma fibronectin from cryoglobulinaemic patients binds to C1q with the same efficiency as plasma fibronectin purified from controls further supports the evidence of structural as well as functional integrity of the molecule as far as opsonic properties are concerned. However the integrity of the gelatin-binding domain of the plasma protein in essential mixed cryoglobulinaemia suggests that the molecule might also efficiently mediate cryoprecipitation of cold insoluble complexes, thereby playing a role in vascular and tissue damage.

Finally, our study also seems to exclude the possibility that an abnormally glycosylated fibronectin molecule, which has been reported in different cell systems and body fluids (47, 48), is produced and released in essential mixed cryoglobulinaemia; using SDS-PAGE, no differences in electrophoretic mobility or apparent molecular mass were observed between plasma fibronectin from patients and controls. However, the possibility cannot be excluded that subtle alterations are present in regions of the polypeptide chain not explored by the monoclonal antibodies employed in this study. Further work is necessary to clarify these issues.

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